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SEXUAL RESPONSES OF ADULT MALE AEDES AEGYPTI USING THE FORCED-COPULATION TECHNIQUE[1, 2]

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Our present knowledge concerning the sexual responses of the male Yellow Fever Mosquito *Aedes* (*Stegomyia*) *aegypti* Linnaeus is based largely on the studies of Roth (1948), Spielman (1964), Jones and Wheeler (1965a, 1965b), Spielman *et al.* (1967), and Craig (1967). According to Roth (1948), adult males in cages copulate repeatedly and remain in a permanent mating state throughout their lives. He observed that one virgin male caged with 16 virgin females for 30 minutes copulated 30 times and made 10 additional attempts to do so. He found that males would copulate with 50-minute-old females. Jones and Wheeler (1965a), using the forced-copulation technique, reported that males would copulate with 4 to 10 virgins within 10 to 20 minutes. Wheeler and Jones (1960) found that while males could be forced to copulate with newly emerged females, they were not inseminated. Lea (1967, 1968) found this to be true for young caged females, and showed that this was influenced by the corpora allata, and was temperature- and strain-dependent. According to Gwadz (1967), females in a cage copulate repeatedly but do not become inseminated until they are 40 to 48 hours old.

VandeHey and Craig (1958) reported that multiple insemination occurred in caged groups of *Aedes aegypti*; Craig (1967) subsequently noted this was true for matings which occurred over a short period of time. Jones and Wheeler (1965b) discovered that males did not force-copulate with inseminated females, and Ludlam (1965) confirmed this. George (1967) reported that while males repeatedly copulate in cages, only the first mating was effective. Craig (1966, 1967) showed that this was due to the male accessory gland component of the semen from the first

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insemination. After implanting male accessory glands into virgin females, Craig obtained a 25% insemination rate using forced-copulation and no insemination with those placed in a cage with fresh males. Spielman *et al.* (1967) reported that when once-mated females were paired with males in a cage 47% of them copulated again, and those which remated very rapidly ejected the semen from the bursae.

It is evident that much remains to be learned about the sexual responses of *A. aegypti* under both forced and cage mating conditions. This paper is concerned with the details of the sexual responses of *A. aegypti* males to very young virgins or to previously copulated females, using the forced-copulation technique, and also includes tests of male potency.

The specific terms which will be used in this paper need to be defined at the outset. *Copulation* refers to the placement of the aedeagus into the upper vagina of the female. *Precoital time* extends from the initial second of the presentation of the male to the beginning of aedeagal emplacement. *Coital time* is the period from the beginning of aedeagal emplacement to its withdrawal from the vagina. *Insemination* is the placement of sperm and male accessory gland materials in the bursa and is followed by spermathecal filling and retention of the male accessory gland material. *Fertilization* is restricted to the placement of spermatozoa in the egg.

Materials and Methods

The mosquitoes of the Bangkok strain were reared at 27° C. and 70% relative humidity. The larvae generally pupated on the sixth and seventh days after hatching. Pupae were sexed by examining their external genitalia with a stereomicroscope at 16 ×, using the criteria of Christophers (1960). Virgin adults of each sex and of known ages were also obtained by removal and separation of the sexes as they emerged. The adults were maintained on 5% sucrose. Test lots of adults were kept either in 28 cm. square wire cages or in standard U. S. pint cartons with a fine net cover on top, and were supplied with sucrose on cotton pads. The induced copulation techniques of McDaniel and Horsefall (1957) and Wheeler (1962) were employed; however, none of the insects were decapitated. All forced-copulations (forced-matings) were with mosquitoes that had recovered from the nitrogen anesthesia which was used in the pinning procedure. Females caged with males (sex ratio 1:1) for 2 days were assumed to be already inseminated when used for subsequent forced-copulation tests, and will be designated as *cage-copulated* or *cage-mated* females. All males were offered only once to a female and then were discarded except in the potency tests. Sperm checks of females were made by examination of the spermathecae and/or the bursa at 430 ×.

Results

1. *Male responses to young virgin females*

Forty-eight-hour-old males were presented to 171 young females of nine precisely known age groups (0, 1, 2, 3, 4, 8, 12, and 24 hours ±1%). The young females often interfered with the copulatory attempts of the males by kicking with their legs (chiefly the metathoracic legs), and when this happened the legs were amputated at the midpoint of the femurs.

TABLE I

Coital times of young virgin Aedes aegypti (L.) females which were force-copulated with virgin males 48 hours old. Cases of prolonged coitus are not included.

Age of females	Inseminated females			Uninseminated females		
	Number of copulations	Coital time, seconds		Number of copulations	Coital time, seconds	
		range	mean		range	mean
0	3	21–724	58.3	1	6	6.0
1 hour	3	18–49	34.3	2	4–6	5.0
2 hours	4	33–70	47.8	2	8–10	9.0
3 hours	3	7–120	55.7	0	—	0
4 hours	6	13–200	62.8	2	6–11	8.5
8 hours	11	40–160	85.1	1	120*	120.0
12 hours**	1	40	40.0	1	16	16.0
12 hours	1	115	115.0	1	4	4.0
16 hours	10	10–134	70.4	1	4	4.0
24 hours**	13	30–398	113.8	2	4–12	8.0
24 hours	9	11–99	31.6	1	7	7.0
	64	7–398	71.4±7.7***	14	4–120	7.5± 1.0***

* Male ejaculated prematurely, datum excluded from mean of total coital time.
** Test performed at two different times.
*** Mean based on total data.

The male responses to these females were either (1) rapid copulation, (2) seizure of the female's cerci and then not copulating, (3) no visible reaction at all, or (4) withdrawal from the female terminalium.

The only visible response of the females to the males was withdrawal of their

TABLE II

Copulation and insemination rates of young virgin female Aedes aegypti (L.) which were force-mated with virgin males 48 hours old

Age of females	Number used	Copulations		Inseminated		Uninseminated		Prolonged coitus	
		Number	%	Number	%	Number	%	Number	%
0	16	4	25.0	3	75.0	1	25.0	0	0
1 hour	15	5	33.3	3	60.0	2	40.0	0	0
2 hours	15	6	40.0	4	66.7	2	33.3	0	0
3 hours	15	4	26.7	3	75.0	0	0.0	1	25.0
4 hours	21	10	47.6	6	60.0	2	20.0	2	20.0
8 hours	16	14	87.5	11	78.6	1	7.1	2	14.3
12 hours*	10	2	20.0	1	50.0	1	50.0	0	0
12 hours	15	3	20.0	1	33.3	1	33.3	1	33.3
16 hours	15	12	80.0	10	83.3	1	8.3	1	8.3
24 hours*	18	15	83.3	13	86.7	2	12.5	0	0
24 hours	15	10	66.7	9	90.0	1	10.0	0	0
	171	85	49.7%	64	75.3%	14	16.5%	7	8.2%

* Test performed at two different times.

terminalia. Very young females emitted droplets from their ani and these often interfered with the male's clasping of the cerci and prevented copulation. This was observed primarily with 0- to 1-hour-old females, and was seldom seen in females 8 or more hours old.

The individual coital times of inseminated females ranged from 4 to 398 seconds, with a mean of 71.4 ± 7.7 seconds, compared to a range of 4 to 16 seconds and mean of 7.5 ± 1.0 seconds for the uninseminated females (Table I). From 20% to 87.5% (85/171 average of 49.7%) of the females force-copulated, and 33.3% to 90% (65/85 average of 75.3%) were successfully inseminated (Table II).

Prolonged forced-copulation was observed with 7 out of 85 pairs of mosquitoes. Most of the males were unable to free themselves at will by even the most strenuous efforts. Only one male, after copulating for 360 seconds, succeeded in freeing himself after struggling for 208 seconds. When the female was dissected 45 minutes later, sperm were seen *only* in the bursa. The other six pairs were *in copula* so tightly that manual stretching tore their abdomens apart rather than separating their terminalia.

TABLE III

Precoital and coital times of force-copulated Aedes aegypti (L.) males in 4 age groups; each male was offered to 15 virgin females of the same age

Age of males	Number used	Number of copulations	Precoital time, seconds		Coital time, seconds	
			range	mean	range	mean
1 Day	10	58	1–171	19.7	3–45	18.5
3 Days	10	68	1–103	16.2	3–66	20.9
5 Days	10	72	1–43	10.2	4–55	20.9
7 Days	10	89	1–105	18.3	4–57	20.3
	40	287	1–171		3–66	
Means		7.2 ± 0.5		16.0 ± 1.2*		20.2 ± 0.7*

* Mean based on total data.

2. *Male potency and variability of response to virgin females*

Adults of precisely known ages (1-, 3-, 5-, and 7-day-old lots ±4%) were used to test the responses of 10 individual virgin males (per lot), each individual being presented in sequence and at known intervals to 15 virgin females, or to as many as would be accepted using the forced-copulation technique. Each of the 4 tests required about 6 hours to complete. The responses of the males were subjectively graded as either (1) feeble, (2) moderate, (3) active, or (4) highly active attempts to copulate, or as (5) withdrawal of their terminalia from those of the females. The first 4 responses were qualitatively assessed by the following activities of the males: (1) the extent of tarsal contact with the female, (2) the degree of flexion of the male's terminalium into the female, (3) the speed and number of attempts to copulate, (4) the forcefulness of cercal clasping, and (5) the extension of the paraprocts with eversion of the aedeagus. Withdrawal of the male's terminalium included a pushing of the female with his tarsi and a flexion of the terminalium away from that of the female.

The precoital times of the 40 males ranged from 1 to 171 seconds, with a mean of 16.0 ± 1.2 seconds. The coital times ranged from 3 to 66 seconds, with a mean of 20.2 ± 0.7 seconds (Table III). The males copulated with 287 out of 600 females (47.8%). The number of females acceptable to individual males ranged from 2 to 15, with a mean of 7.2 ± 0.5.

Most of the females were not accepted sequentially by the males; only one out of the 40 males used accepted all 15 females, and this individual did not accept 2 additional females. This highly potent male's initial precoital and coital times were 21 and 24 seconds, respectively. His final precoital and coital times were 3 and 4 seconds, respectively. In these experiments, 6 or more females were accepted sequentially by 19 out of 40 males (47.5%), and 8 or more females were accepted sequentially by 7 out of 40 males (17.5%). Only 2 out of 40 males (5%) copulated sequentially with less than 3 or more than 10 females.

The age of the mosquitoes apparently did not influence either the precoital or coital times (Table III). The number of copulations by 5- and 7-day-old males was significantly higher than that by 1- and 3-day-old males (p less than 0.01). The increase in the number of copulations of 3-day-old males *versus* 1-day-old males and of 7-day-old males *versus* 5-day-old males was significant (p less than 0.01).

TABLE IV

Responses of Aedes aegypti (L.) males in 4 age groups; each male was offered to 15 virgin females of the same age

Male age	Withdrawal responses	Coital responses					Total responses
		Feeble	Moderate	Active	Highly active	Row totals	
1 Day	5	3	29	15	11	58	63
3 Days	13	20	22	15	11	68	81
5 Days	1	30	24	13	5	72	73
7 Days	3	28	28	23	10	89	92
	22 7.1%	81 28.2%	103 35.9%	66 23.0%	37 12.9%	287 92.9%	309

Presentation of a virgin female to a male resulted in definite responses to the female in 309 out of 600 males (52%). In the other 287 (48%), the males showed no recognizable response to the females. Of the responses that did occur, 7.1% (22/309) were withdrawals and 92.9% (287/309) were coital responses. The degree of the coital responses were as follows: feeble, 28.2% (81/287); moderate, 35.9% (103/287); active, 23.0% (66/287); and highly active, 12.9% (37/287) (Table IV).

3. *Forced copulation of virgin versus cage-mated females*

One hundred virgin and 100 cage-mated females were each individually offered a series of one to 5 males, in 5 lots of 20, using the forced-copulation technique. If copulation occurred, no additional males were presented. All the mosquitoes were 48 hours old ±3 hours.

Among the virgins, 95% force-mated for 6 to 76 seconds, with means for the 5 replicates ranging from 22.8 to 29.9 seconds, and with an overall mean of 26.7 ± 1.3 seconds; 81 out of 95 virgins (85.3%) accepted the first male offered, and 10 out of 95 accepted the second male (10.5%).

In great contrast to the above, only one out of the 100 cage-mated females could be force-copulated, and this one did so for only 3 seconds.

Fifty of those virgin females which had previously been successfully force-copulated were again presented to virgin males for a second time, 12 hours after the initial force-copulation. Only 5 out of 50 of them (10%) force-copulated a second time. Their coital times ranged from 26 to 55 seconds with a mean of 28.8 ± 6.8 seconds. However, 48 out of 50 concurrent virgin controls (96%) force-copulated. The coital times of these controls ranged from 12 to 58 seconds, with an overall mean of 25.0 ± 1.3 seconds.

Discussion

Aedes aegypti females which are less than 24 hours old can be inseminated by force-mating (this paper), but none are inseminated at this age within a cage (Lea, 1967, 1968; Gwadz, 1967). Spielman *et al.* (1967) suggested that (1) semen is altered during or immediately after insemination of virgin females and that (2) semen is lost, if not so altered. Thus, it is possible that very young females mating in a cage do not yet have the ability to alter semen, and therefore do not retain it. Since the aedeagus blocks the genital orifice during forced-mating about 5 times longer than during cage-mating, it is possible that the retention of the semen in young force-mated females is due to the fact that it has time to become self-altered, whereas the semen in young cage-mated females flows out of the bursae before it has time to become self-altered. It is known that the male accessory gland material alone gels very rapidly in saline *in vitro* (Jones and Wheeler, 1965a). Since Jones (unpublished data) noted that the bursal walls lack the machinery of secretory cells, the bursa probably does not secrete anything which would alter the semen.

It is also conceivable that the bursae of very young females differ in several respects from those of older females. The very young females are swollen by air and fluids in the gut (Christophers, 1960) and this might distort the bursae. If during the relatively prolonged period of forced-mating, the pairs were more firmly united than during the briefer period of cage-mating, then a longer and firmer union might permit the force-copulating male to fill the bursa of a very young female, whereas a shorter and less firm union might not permit the free male to do so adequately.

Further, there may be a difference in the coital mechanics between forced-copulation and cage-mating of the young females. The descriptions of coitus by Jones and Wheeler (1965b) and Spielman (1964) differ enough to suggest that this is a very real possibility. The male may be unable to grasp the young female properly in cage-mating but can do so when force-copulated. If the cage-copulating male does not grasp the young female correctly, then (1) the male may only appear to cage-copulate with the young female but not ejaculate, or (2) the aedeagus of the free male may not be able to attach to the dorsal vaginal valve properly so that the ejaculate is not deposited in the bursa. The phallotreme's position must be exactly within the coital cavity of the inverted upper vagina or the female will not be inseminated properly (Jones and Wheeler, 1965b).

The occurrence of prolonged coitus in cage populations of *Ae. aegypti* has never been reported, yet it was observed in 7 out of 85 (8.2%) force-copulations with young females. This phenomenon may provide a means of determining if the aedeagus blocks the opening to the vestibule (the funnel through which sperm enter the spermathecal ducts). The factors involved in the termination of coitus are not well known. It is believed that the aedeagus overextends and that the connection with the dorsal vaginal valve is easily broken. The aedeagus is then free and pushes against the male's own anal cone. The female then dislodges the male with her legs (Jones and Wheeler, 1965b). In prolonged forced-coitus, the gelation of the male accessory gland material might hold the aedeagus *in situ*.

Very evident in this study was the high degree of variability of the sexual responses of males that had been reared under nearly identical conditions and which were given presumably similar stimuli. Sometimes a male would not copulate with one virgin female but would with a different female. The coital responses shown by individual males appeared to vary between females. Some individual males are certainly more potent in that they are highly active and will copulate sequentially more than 6 times. Although it could be argued that a series of virgin females differ substantially in their attractiveness to a given male, this seems unlikely. Often, in separate tests, females that were actively refused (as indicated by withdrawal of the male's terminalium) by different males were subsequently acceptable to other males. The mean precoital times of 16.0 ± 1.2 seconds for forced-copulation are short compared to the means of 34.1 ± 4.2, 45.0 ± 4.7, and 47.5 ± 6.5 seconds reported for mating in lantern chimneys (Spielman *et al.*, 1967). In the latter study, the time required for flight and tarsal contact probably accounts for the longer interval. The coital time (mean 20.2 ± 0.7 seconds) of the males repeatedly force-copulated agrees with the 19.7 ± 6.4 seconds mean reported by Spielman (1964) for cage-copulated males that had been previously mated twice.

The mean coital time of 26.7 ± 1.3 seconds for 48-hour-old virgins that were force-copulated is higher than the means of 13.7 ± 3.2 seconds (Spielman, 1964), 17.6 ± 0.5)Spielman *et al.*, 1967), and 13.2 seconds (Jones and Wheeler, 1965a) for 3- to 7-day-old virgins that were cage-mated.

The mean of 25.3 ± 0.8 seconds of Ludlam (1965) and the mean of 26.7 ± 1.3 seconds in this study for coital times by forced-copulation agree; they differ from the longer mean of 31.3 ± 1.6 seconds reported by Jones and Wheeler (1965a).

This study strikingly supports the hypothesis that in the Bangkok strain of *Ae. aegypti,* cage-mated females cannot be subsequently force-copulated with males. It was not possible to force-copulate 99 out of 100 cage-mated females. This poses the question of whether the male can distinguish an inseminated from a virgin female. Clasping reflexes were generally present but aedeagal contact was never made with the cage-mated females when the forced-copulation technique was used. Ludlam (1965) did not observe even the clasping reflexes with males of this strain. Although it is conceivable that chemical stimulus is involved here, there is no evidence to support this. On the other hand, there is a possible morphological difference between virgin and previously copulated females. In the latter, both the cerci and the eighth abdominal segment appeared to be more deeply retracted, and this would make it difficult for the male to seize the cerci with his claspers. This would not account for those cases where the male actively withdrew his terminalium from the female. The work of Spielman *et al.* (1967) differed from these findings, since they were able to force-copulate previously cage-mated females.

Since most (95/100) virgin females which are 48 to 60 hours old are readily force-copulated by males of the same age, an optimum standard for evaluating the behavior of controls was established. Although 10.0% (5/50) of those females which had force-copulated 12 hours earlier did so again, it is possible that this seemingly high value is due to the fact that some of these females were not initially inseminated. More data are needed on insemination rates obtained with the forced-copulation technique.

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SUMMARY

1. Virgin females which are 24 hours old or less interfered with the copulatory attempts of males by kicking with their legs and emitting fluid from their ani. About 50% of these young females could be force-copulated, and 75% of these were inseminated.

2. The mean forced-coital time of young virgin females which were inseminated was 71.4 seconds. Those which were not inseminated force-copulated for a much shorter time (15.6 seconds).

3. The sexual responses of males to young virgin females were not uniform with the forced-copulation technique. Active refusal by withdrawal of the male terminalium from the female by the male was more definite with young females than with older ones.

4. Prolonged coitus was observed in about 8% of the forced-copulations with young virgin females; this phenomenon has not been reported for cage-matings nor has it been seen in nature.

5. Ninety-five of 100 virgin females force-copulated, whereas only one 3-second copulation was obtained with 100 females that had been previously cage-mated.

6. A force-copulation rate of 10% was recorded for females that had force-copulated 12 hours previously.

7. The cerci and the eighth abdominal segment appeared to be more deeply retracted in inseminated females than in virgin females.

8. While individual males varied in their sexual responses toward similar females, 52% of them definitely responded to them and 92% of these individuals copulated.

9. Using the forced-copulation technique, precoital and coital time means were 16.0 and 20.7 seconds, respectively. The age of the male did not influence these values.

10. The number of copulations by 5- and 7-day-old males was significantly higher than that by 1- and 3-day-old males.

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